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Brassica napus DNA markers linked to white rust resistance in *Brassica juncea*

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Abstract White rust, caused by *Albugo candida*, is an economically important disease of *Brassica juncea* mustard. The most efficient and cost effective way of protecting mustard plants from white rust is through genetic resistance. The development of canola quality *B. juncea* through interspecific crosses of *B. juncea* with *Brassica napus* has lead to the introgression of white rust resistance from *B. napus* into *B. juncea*. The objective of this study was to identify DNA markers for white rust resistance, derived from the introgressed *B. napus* chromosome segment, in a BC_3 F_2 population of condiment *B. juncea* mustard. This segregating population was phenotyped for white rust reaction and used to screen for AFLP markers associated with white rust resistance using bulked segregant analysis. Segregation data indicated that a single dominant gene controlled resistance to white rust. Eight AFLP markers linked to white rust resistance were identified, all derived from *B. napus*. The *B. napus* chromosome segment, carrying the white rust resistance gene ($Ac2V_1$), appeared to have recombined with the *B. juncea* DNA since recombinant individuals were identified. Comparative mapping of the eight *B. napus*-derived AFLP markers in a typical *B. napus* mapping population was inconclusive; therefore, the size of the introgressed *B. napus* fragment could not be determined.

Keywords *Brassica juncea* · AFLP markers · *Albugo candida* · White rust resistance

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Introduction

White rust, caused by *Albugo candida* (Pers.) Kuntze is a widespread and destructive disease of cruciferous crops, mustard [*Brassica juncea* (L) Czern. and Coss.] and turnip rape (*Brassica rapa* L.) (Walker and Williams 1973; Saharan and Verma 1992; Buzza 1995; Kole et al. 1996). In contrast, Canadian cultivars of *Brassica napus* canola are highly resistant or immune to white rust. Yield losses of 30 to 60% have been reported in *B. rapa* due to white rust infection in China (Liu et al. 1996). Physiological specialization in *A. candida* is classified on the basis of specificity to different species of crucifers (Hill et al. 1988). However, host specificity in *A. candida* is not an absolute adaptation to a particular species, especially when the races are from hosts sharing a common genome (Liu et al. 1996). The predominant race of *A. candida* on *B. juncea* in western Canada is race 2A and Canadian oriental mustard cultivars Domo, Cutlass and AC Vulcan have some resistance to this race. Another variant of race 2 (race 2V), for which no natural resistance in *B. juncea* has been identified, was virulent on the race 2A-resistant cultivar Cutlass (Rimmer et al. 2000). Resistance to white rust in *Brassica* species, for which information is available, is governed by simple Mendelian inheritance; for example, a single dominant gene in *B. juncea* controls resistance against race 2 (Tiwari et al. 1988; Rimmer and Buchwaldt 1995), three dominant genes in *B. napus* control resistance against race 7 (Fan et al.1983; Liu et al. 1996) and a single dominant resistance gene in *B. rapa*, against race 2A, has been reported (Kole et al. 1996).

The development of marker-assisted selection (MAS) strategies for resistance to white rust will be valuable in identifying resistant plants from among segregating populations. DNA-based tests can replace more time-consuming pathology testing and thus the analysis of more plants. A MAS breeding strategy for the development of white rust-resistant cultivars would be useful in both condiment mustard and canola quality *B. juncea* breeding programs. Currently, markers are available for the selection of *B. juncea* plants carrying the resistance gene ($Ac2A_1$) to *A. candida* race 2A (Prabhu et al. 1998).

To develop canola quality *B. juncea* with zero erucic acid, low glucosinolate and reduced linolenic acid, interspecific crosses between *B. juncea* and *B. napus* were made to introgress the modified fatty acid composition traits of *B. napus* into *B. juncea*. In doing so, resistance to white rust also was transferred to *B. juncea*, and the current study focuses on developing DNA markers for this novel *B. napus* locus (*Ac2V$_1$*) in a *B. juncea* genetic background.

Materials and methods

Plant material

Resistance to white rust was introgressed from *B. napus* canola into *B. juncea* through interspecific crosses. The zero erucic acid, low glucosinolate (non-allyl) *B. juncea* line J90-4253 (Rancy et al. 1995) was crossed with the low linolenic acid, *B. napus* line S86-69 from the University of Manitoba, Winnipeg. Interspecific F_1 plants were backcrossed to J90-4253 to produce BC_1 F_1 seed. BC_1 F_1 plants were grown and crossed with the *B. juncea* oriental mustard cultivar AC Vulcan to produce BC_2 F_1 seed, from which BC_2 F_1 plants were grown and crossed with AC Vulcan to produce BC_3 F_1 seed. BC_3 F_1 seeds were half-seed selected for normal erucic acid content (about 25%) and only these were grown into plants in the greenhouse. This was followed by leaf glucosinolate analysis, and plants that contained allyl glucosinolate were selected. These condiment quality mustard plants were then inoculated with a single pustule isolate of *A. candida* race 2V, the virulent *B. juncea* specific race of this fungus, scored for disease reaction and white rust-resistant plants were identified and selected. Two BC_3 F_1 plants, resistant to white rust, were self-pollinated and BC_3 F_2 seed was produced. BC_3 F_2 seedlings were evaluated for *A. candida* race 2V disease reaction, and plants free from disease were self-pollinated to produce BC_3 F_2 seed. BC_3 F_2 families were then grown (12 plants from each family), evaluated for the *A. candida* race 2V disease reaction to determine homozygosity for white rust resistance of BC_3 F_2 plants.

White rust evaluation

A single pustule isolate of race 2V of *A. candida* was increased by inoculation on the susceptible cultivar Cutlass (*B. juncea*) and mature zoosporangia were collected in gelatin capsules (Parke-Davis Size 00) and stored in glass screw-cap vials at −10 to −20 °C. Inoculum was prepared according to the methods of Liu et al. 1996. Briefly, zoosporangia (one capsule) were placed in a 125 ml Erlenmeyer flask containing 30 ml of distilled water, sealed with Parafilm, and shaken gently. Sporangia were incubated at 12 °C for 3 to 4 h for the induction of zoosporogenesis, and then placed on ice to avoid zoospore encystment. The number of zoospores were quantified and adjusted to 1×10^4 zoospores ml^{-1}.

Seedlings were planted in 12-cell multipots and maintained in a growth room with a day/night temperature of 22/17 °C and a 16-h photoperiod. Seedlings (6–7 days after planting) were inoculated by applying 10μ of a zoospore suspension with an Eppendorf repeater pipette to each side of each cotyledon and 10μ to the apical meristem. Inoculated seedlings were covered with plastic and incubated in a refrigerated chamber at 15 °C in the dark for 24 h before returning them to the growth room.

Disease reactions were scored on a 0–9 scale, 8–10 days after inoculation (Williams 1985). Cotyledons and first leaves, which showed no symptoms or only small necrotic flecks with no sporulation, were scored as IP 0, 1 or 2, depending on the extent of necrosis, and these were considered resistant. Cotyledons and leaves, which showed scattered or coalescing pustules on either or both the abaxial or adaxial surface, were scored as IP3 or greater and considered susceptible. The plant populations used in this study showed only IP 0 or 1 (highly resistant) and IP 8 or 9 (highly susceptible) individuals. No intermediate IPs were observed.

DNA extraction and AFLP analysis

Small leaf samples from 5 to 10 plants of each parental line were bulked, lyophilized, and ground to a powder with liquid nitrogen in a mortar and pestle. Prior to *A. candida* inoculation, small leaf samples were also collected from individual BC_3 F_2 seedlings into 1.5-ml microtubes, then lyophilized and ground by shaking with glass beads.

DNA extraction was performed with 30 to 50 mg of dry, ground tissue in 1.5-ml microtubes using the "DNeasy" plant extraction kit (Qiagen) according to the manufacturer's instructions. Final DNA concentrations were set at 25 ng/μl in water.

The AFLP analysis was conducted using a kit (Gibco BRL, Mississauga, Ontario) according to the manufacturer's instructions. The AFLP procedure essentially follows that first described by Vos et al. (1995). Detection of the AFLP fingerprints included 5′ end-labelling of the *Eco*RI selective primer with gamma-P^{33} ATP, electrophoresis of PCR products in 4% acrylamide (1 × TBE) gels followed by autoradiography of the dried gels.

Bulked segregant analysis (BSA)

A total of 64 selective primer combinations, each primer included three selective nucleotides, were screened using the BSA strategy (Michelmore et al. 1991). The BSA included DNA of the parents, *B. juncea* cultivars AC Vulcan (white rust race 2V-susceptible) and *B. napus* line S86-69 (white rust-resistant). The two bulked segregants were prepared by combining equal amounts of DNA from each of eight white rust-resistant and nine white rust-susceptible BC_3 F_2 seedlings derived from a single BC_3 F_1 plant (#2535). The BC_3 F_2 white rust-resistant seedlings used in the bulk were homozygous for resistance according to the BC_3 F_3 disease reaction data.

Linkage analysis

The white rust race 2V resistance locus was designated as *Ac2V$_1$*. Linkage between the *Ac2V$_1$* locus and AFLP markers was established with Mapmaker/exp V3.0 software (Lander et al. 1987; Lincoln et al. 1992) by analysing marker segregation data in both BC_3 F_2 populations (#2535, #2534) as well as in the combined BC_3 F_2 population #2535 and #2534. AFLP markers and the *Ac2V$_1$* locus were grouped using a minimum LOD threshold of 2.5 and a maximum recombination fraction of 0.3 as linkage criteria.

Results

The disease reaction of seedlings to *A. candida* race 2V was rated on a 0 to 9 scale which considered the number, size and quality of lesions and pustules formed on inoculated cotyledons and leaves. *B. napus* variety Apollo and *B. juncea* landrace Common Brown were included in the seedling tests to provide a reference for highly resistant (score 0) and highly susceptible (score 8, 9) seedlings, respectively. The two BC_3 F_1 seedlings that were selfed were each rated highly resistant. The derived BC_3 F_2 and BC_3 F_3 seedlings were all rated as either highly resistant (score 0, 1) or highly susceptible (score 8, 9). No intermediate disease reactions were observed in the study and thus the resistance to *A. candida* race 2V was analyzed as a qualitative trait.

The BC_3 F_3 seedling disease reaction data was used to classify the BC_3 F_2 plants as true breeding (homozygous) or segregating (heterozygous). The BC_3 F_2 population segregation data showed that the two BC_3 F_2 populations each segregated in a 1:2:1 ratio ($\chi^2_{0.05} = 0.69$ and 2.41). The entire population of 73 seedlings also segregated in a 1:2:1 ratio ($\chi^2_{0.05} = 2.73$) (Table 1).

Table 1 Phenotypic and genotypic segregation of BC_3 F_2 *B. juncea* seedlings carrying a *B. napus*-derived *A. candida* race 2V resistance locus

Population	Resistant		Susceptible homozygous	*n*	χ^2 (1:2:1)$_{0.05}$
	Homozygous	Heterozygous			
2535	8	22	9	38	0.69
2534	5	21	8	34	2.41
All	13	43	17	73	2.73

Table 2 *B. napus* AFLP markers linked to $Ac2V_1$ incorporated into *B. juncea* by interspecific crossing with *B. napus*

Marker[a]	Selective primers[b]	
	*Eco*RI	*Mse*I
E1M2e	AAG	CAG
E2M2l	AAC	CAG
E1M5c	AAG	CTA
E7M3a	ACC	CAG
E2M2b	AAC	CAG
E5M2b	ACA	CAG
E2M3a	AAC	CAC
E1M2d	AAG	CAG

[a] Lower case letters refer to bands within the AFLP profile
[b] Three nucleotides on the 3' end of selective primers published by Vos et al. (1995)

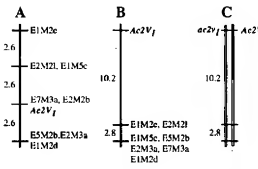

Fig. 1 Genetic linkage maps of AFLP markers and the $Ac2V_1$ locus derived from *B. napus* and introgressed into *B. juncea*. A *B. juncea* BC_3 F_2 population 2535, B *B. juncea* BC_3 F_2 population 2534, C Schematic diagram of the $Ac2V_1$ interval in the white rust-resistant *B. juncea* plant 2534–35 showing the $Ac2V_1$ locus to be heterozygous. *Black and white chromosome segments* represent *B. napus* and *B. juncea*, respectively

Bulked segregant analysis of population 2535, using AFLP markers, resulted in a total of 22 candidate markers being amplified. There were 19 candidate markers amplified from *B. napus* S86-69 and the white rust-resistant bulk and three candidate markers amplified from AC Vulcan and the susceptible bulk. Eight of the 19 *B. napus* markers (Table 2) showed linkage to a single resistance gene in population 2535, and maintained this grouping at LOD 7.4 (Fig. 1A). Seven of the *B. napus* markers formed a very similar linkage group, with population 2534 (Fig. 1B). The two linkage maps (Fig. 1A, B) showed marginally different marker orders in relation to the resistance gene locus, placing the $Ac2V_1$ in a terminal position or alternatively in the middle of the linkage group (Fig. 1A, B respectiveley). The interval of the *B. napus*-derived markers was similar (13-cM, population 2534; 7.8-cM, population 2535) between the two linkage maps. There was evidence of recombination between the $Ac2V_1$ gene and *B. napus* AFLP markers in both BC_3 F_2 populations.

There was one heterozygous resistant BC_3 F_2 individual in population 2534 (plant #35) with a recombination event between $Ac2V_1$ and the first markers E1M2e and E2M2l. Figure 1C shows the predicted chromosome interval surrounding the $Ac2V_1$ gene of this single individual. Another two BC_3 F_2 plants from population 2534, also with recombination events in this interval, were susceptible to white rust.

Discussion

The resistance to *A. candida* race 2V, introgressed from *B. napus* to *B. juncea*, appeared to be controlled by a single, dominant gene. The two BC_3 F_1 seedlings used to develop the BC_3 F_2 populations were both fully resistant to white rust and the two BC_3 F_2 populations segregated phenotypically 1 homozygous resistant: 2 heterozygous resistant: 1 homozygous susceptible (Table 1). This is consistent with resistance to *A. candida* in other studies where a single, dominant gene was identified for resistance to *A. candida* race 2A in *B. juncea* (Tiwari et al.1988; Rimmer and Buchwaldt 1995) and *B. rapa* (Kole et al. 1996). If multiple genes that controlled resistance to *A. candida* race 2V are present in *B. napus*, it is possible that the genes would not all be introgressed into *B. juncea* AC Vulcan via the crossing scheme used. White rust-resistance genes present in the A genome of *B. napus* stand a reasonable chance of recombining and being introgressed into the *B. juncea* A genome, while C-genome white rust resistance genes of *B. napus* would rarely be transferred into *B. juncea* due to the very low frequency of pairing observed between the C-genome chromosomes of *B. napus* with the B-genome chromosomes of *B. juncea* (Attia and Robbelen 1986; Attia et al. 1987).

The BC_3 F_1 seedlings were either fully susceptible or resistant to white rust under artificial disease conditions, which suggested that the white rust resistance allele from *B. napus* is dominant over alternate alleles and increases the frequency of white rust-resistant plants in breeding populations.

We identified 22 candidate AFLP markers through BSA after screening 64 different AFLP selective primer combinations. The vast majority of these AFLP fragments were derived from *B. napus* which suggests: (1) that the segment of the *B. napus* genome introgressed into *B. juncea* is large, and/or (2) the orthologous *B. napus*

and *B. juncea* segments are highly polymorphic. There was recombination between the *B. napus* AFLP fragments that were linked to the $Ac2V_1$ gene which suggests that the *B. napus* chromosome segment is incorporated into the *B. juncea* genome, most likely the A genome, and is involved in pairing during meiosis. Recombination distances calculated in the two BC_3F_2 populations (2535, 2534) were very consistent, but it is difficult to determine if similar recombination distances would be observed for this *B. napus* segment in a typical *B. napus* mapping population. Presumably, the recombination distances are underestimated in Fig. 1A, B due to reduced pairing of the *B. napus* segment with the orthologous *B. juncea* segment. The analysis using Mapmaker V3.0 and the map distances should therefore be considered as estimates.

The precise marker order was not determined since the two BC_3F_1 plants (2535 and 2534) appear to have generated sufficiently different gamete populations and, thus, genetic maps. In one case (2535), the $Ac2V_1$ gene is the terminal locus whereas the 2534 map showed the $Ac2V_1$ gene flanked by *B. napus*-derived markers (Fig. 1A, B). In general, there are similar clusters of co-segregating markers and similar map distances between both genetic maps. From a practical standpoint, the suite of markers show tight linkage to the $Ac2V_1$ gene and precise marker order may not be necessary for developing marker-assisted breeding strategies with these markers.

There were 72 BC_3F_2 plants that were genotyped with the seven AFLP markers derived from *B. napus*. Three of these plants showed a recombination event in the first interval between $Ac2V_1$ and the first neighbouring markers E1M2e and E2M2l. One of these three recombinant plants was resistant (heterozygous) to infection with race 2V, and the recombination is depicted in Fig. 1C. The BC_3F_1 family from this BC_3F_2 plant was segregating and, therefore, it should be possible to select BC_3F_3 plants that are homozygous for resistance to white rust with this reduced segment of the *B. napus* chromosome. With further marker saturation in this interval and continued backcrossing with plants from this selected BC_3F_3 family, it would be possible to retrieve plants with a reduced *B. napus* DNA segment containing the $Ac2V_1$ gene.

In summary, we have reported on a unique source of resistance for white rust in *B. juncea* which was intro-gressd into *B. juncea* from an interspecific cross with *B. napus*. The disease resistance is very strong, with current elite lines of both condiment mustard and canola quality *B. juncea* showing a complete absence of white rust infection under field and greenhouse conditions. The intro-gressed segment of *B. napus* has been tagged by AFLP markers which will be useful in developing high-throughput MAS strategies. The *B. napus* resistance may also be transferable through interspecific crosses and MAS, to *B. rapa*, which also shows high levels of susceptibility to white rust.

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